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Production of melanin by *Aspergillus fumigatus*

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Melanins, or melanin-like compounds, may play a role in the pathogenesis of a number of human fungal infections. This study investigated the production of melanin by the important opportunistic pathogen *Aspergillus fumigatus*. Conidia from *A. fumigatus* were harvested and treated with proteolytic enzymes, denaturant and hot, concentrated acid; this yielded dark particles which were similar in size and shape to the original propagules. Electron spin resonance spectroscopy revealed that the conidial-derived particles were stable free radicals consistent with an identification as melanin. Melanin particles were used to immunize BALB/c mice in order to produce a total of five anti-melanin monoclonal antibodies (mAbs). The latter mAbs were strongly reactive both with intact conidia and with extracted melanin particles by ELISA and immunofluorescence reactivity. Immunofluorescence labelling with the novel mAbs was used to examine the temporal expression of melanin during *in vitro* culture of *A. fumigatus* – melanization was confined to conidial structures and was absent from hyphae. SDS-PAGE L-3,4-dihydroxyphenylalanine (L-DOPA) substrate analysis confirmed the presence of a laccase-type activity in conidial extracts, but not in hyphae. Melanin-binding mAbs were used to detect the presence of melanized conidia in three patients with nasal aspergilloma, indicating that *in vivo* melanization may occur during infection.

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INTRODUCTION

Aspergillus fumigatus is the most ubiquitous of the airborne saprophytic fungal pathogens in immunocompromised patients in developed countries (Latgé, 1999). It is the causative agent of various disease states in humans including allergic bronchopulmonary aspergillosis, aspergilloma and invasive pulmonary aspergillosis (Tomee & van der Werf, 2001) – the latter is a frequent cause of morbidity and mortality in immunocompromised individuals. The ability of *A. fumigatus* to cause disease is presumably related to its possession of various virulence traits given the fact that other members of the genus *Aspergillus* are either less pathogenic or non-pathogenic. Several putative virulence determinants of *A. fumigatus* have been examined, including adhesins, pigments, toxic molecules and enzymes (Latgé, 1999, 2001; Langfelder *et al.*, 1998).

Many fungi produce melanins, which are dark-brown or black pigments formed by the oxidative polymerization of phenolic compounds. Melanin synthesis is associated with virulence in a number of human pathogenic fungi, such as *Cryptococcus neoformans* (Nosanchuk *et al.*, 2000; Rosas *et al.*, 2000a,b; Casadevall *et al.*, 2000) and *Sporothrix schenckii* (Romero-Martinez *et al.*, 2000; Morris-Jones *et al.*, 2003). Melanin has also recently been identified in *Paracoccidioides brasiliensis* (Gómez *et al.*, 2001) and *Histoplasma capsulatum* (Nosanchuk *et al.*, 2002). Melanin can protect *C. neoformans* against the effects of amphotericin B, macrophage-mediated phagocytosis, nitrogen-derived and oxygen-derived oxidants, microbial peptides and ultraviolet light (Hamilton & Holdom, 1999). In *C. neoformans*, melanization has been shown to occur during human infection (Nosanchuk *et al.*, 2000).

A. fumigatus conidia are known to produce a bluish-green pigment by using the dihydroxynaphthalene (DHN)-melanin pathway (Tsai *et al.*, 1997, 1998, 1999; Langfelder *et al.*, 1998; Watanabe *et al.*, 2000). Several studies have shown that conidia lacking pigmentation due to the defective polyketide synthase gene *pksP* are less resistant to monocyte attack *in vitro*; they also show reduced virulence in animal models (Jahn *et al.*, 1997; Langfelder *et al.*, 1998; Tsai *et al.*, 1998, 1999). In addition, Jahn *et al.* (2000) showed that loss of conidial pigment, in both *A. fumigatus* and *Aspergillus niger*,

Abbreviations: DHN, dihydroxynaphthalene; DOPA, 3,4-dihydroxyphenylalanine; ESR, electron spin resonance; IF, immunofluorescence; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

is linked with an increased susceptibility to reactive oxygen species released from human polymorphonuclear leukocytes (PMNs) and monocytes.

However, there is little available information of the precise physio-chemical nature of the *A. fumigatus* pigment or on its temporal expression during culture and during infection. In this report, we confirm the presence of melanin in conidia and describe the production of new melanin-reactive monoclonal antibodies (mAbs) for use in the definition of melanization *in vitro* and during human infection.

METHODS

Fungal strains and media. *A. fumigatus* B5233 was obtained from Dr K. J. Kwon-Chung (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). *A. fumigatus* NCPF (National Collection of Pathogenic Fungi) 2010 and NCPF 2078 were provided by Dr Mary Holdom, of Guys' Hospital, Kings College, London, UK. *A. fumigatus* was maintained by monthly subculture on Sabouraud dextrose agar slants (SDA) (Oxoid). *A. fumigatus* was grown on SDA at 37 °C for 5 days and the conidia were collected by adding 5 ml of sterile PBS to the culture plates; conidia were removed by gentle scraping with a plastic loop. The conidia were collected by centrifugation at 8000 *g* for 30 min and were then washed three times with sterile PBS.

Sabouraud broth (Oxoid) was used for culturing *A. fumigatus* in the mycelial form at 37 °C for 5 days with continuous shaking at 120 r.p.m. To harvest the mycelia, the broth cultures were filtered through sterile Whatman paper; mycelia were then scraped from the paper and washed three times with sterile PBS.

Preparation of slide cultures of *A. fumigatus*. Slide cultures were prepared in sterile Petri dishes by placing slides on sterile glass rods. SDA blocks cut into 1 cm squares were put on the slides and a small amount of the fungus was inoculated to each of four sides of the agar block. A sterile cover slip was then placed on the top of the inoculated agar block. Plates were then sealed with masking tape and kept at room temperature for 2 weeks. Slides were periodically removed from the Petri dish and examined. When fungal conidiation was complete, the cover slips and agar blocks were removed and absolute ethanol was added to the slides which were then left until dry.

Isolation and purification of melanin-like particles from *A. fumigatus* conidia. *A. fumigatus* B5233 was grown on SDA plates for 5 days at 37 °C and conidia were collected as described. Conidia were washed three times with PBS, with a final wash with 1·0 M sorbitol and 0·1 M sodium citrate (pH 5·5). Novozyme (cell-wall-lysing enzymes from *Trichoderma harzianum*; Sigma) was added at 10 mg ml^{-1} and incubated overnight at 30 °C to generate protoplasts. The protoplasts were collected by centrifugation, washed three times with PBS and incubated in 4·0 M guanidine thiocyanate (denaturant; Sigma), overnight at room temperature. Dark particles were collected by centrifugation. These were then washed three times with PBS and treated with 1·0 mg ml^{-1} Proteinase K (Roche Laboratories) in reaction buffer (10·0 mM Tris, 1·0 mM $CaCl_2$ and 0·5 % SDS, pH 7·8) and incubated at 37 °C. Debris was washed three times with PBS and boiled in 6·0 M HCl for 1·5 h. After treatment by boiling in acid, melanin particles were collected by filtration through Whatman paper and washed extensively with distilled water. Particles were then dialysed against distilled water for 10 days until the acid was completely removed and were then lyophilized as appropriate.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Briefly, for TEM, the melanin particles were fixed in 2 % (v/v) glutaraldehyde for 2 h, then incubated overnight in 4 % (v/v) formaldehyde/1 % (v/v) glutaraldehyde/0·1 PBS and subjected to 1·5 h post-fixation in 2 % (w/v) osmium. Dehydration was accomplished by serial incubation in graded ethanol and two final incubations in 100 % ethanol. The melanin particles were embedded in Spurr's resin. TEM pictures were then obtained with a Hitachi H 7600 transmission electron microscope. For SEM, the melanin particles were fixed overnight in a 4 % glutaraldehyde solution in PBS, transferred on polylysine-coated coverslips, dehydrated by incubation in graded ethanol, mounted with gold–palladium and viewed in a Hitachi S-3500 N scanning electron microscope.

Electron spin resonance (ESR) spectroscopy analyses. ESR spectroscopy has been used to study and define melanins based on the properties of unpaired electrons present in melanin (Enochs *et al.*, 1993) by using a Gunn diode as a microwave source. A total of 2 g freeze-dried material was used in each case (analysis was carried out in silica cuvettes). *A. niger* melanin was used as a positive control.

Immunization protocol. Two 6- to 8-week-old female BALB/c mice were immunized with an intraperitoneal injection of melanin particles extracted from *A. fumigatus* conidia suspended in a 1 : 1 (v/v) emulsion of incomplete Freund's adjuvant (Difco) and PBS, followed by an identical inoculation at weeks 3 and 6 after the initial immunization. The mice were bled from the tail vein 4 days after the week 6 inoculation and their sera were analysed for antibodies to melanin by ELISA, as described below. The mouse with the highest antibody titre to melanin received a final melanin immunization before being used to generate hybridomas.

Production of hybridomas. Spleen cells from the chosen mouse were fused to sp2/0 myeloma cells at a ratio of 10 : 1 in the presence of 50 % (v/v) polyethylene glycol to generate hybridomas as described previously (Zola & Brooks, 1982; Hamilton *et al.*, 1990a, b). Supernatants were screened for the presence of mAbs to melanin by ELISA (see below).

Melanin ELISAs. A suspension of 50 μg of melanin from *A. fumigatus* in distilled water was plated in each well of a polystyrene 96-well ELISA plate (Corning Glass Works) and incubated overnight at room temperature till dry. The melanin was then heat-fixed to the polystyrene solid-phase support by incubating the plates at 60 °C for 30 min. Wells were blocked to prevent non-specific binding by adding 200 μl of 5 % (w/v) bovine serum albumin (BSA) in PBS at 4 °C for overnight. The plates were washed three times with 0·1 % (v/v) Tween 20 in Tris-buffered saline (TBS). Sera (from mice inoculated with melanin) or antibodies (i.e. mAbs) were diluted in 1 % (w/v) BSA in PBS and added (100 μl) to the wells of the melanin-coated plates and incubated for 1·5 h at 37 °C. After three washes, 100 μl of a 1 : 1000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-mouse immunoglobulin M (IgM) (Jackson) in PBS was added to the wells and incubated for 1·5 h at 37 °C. Plates were then washed, and 100 μl of *o*-phenylenediamine in 0·01 M sodium citrate buffer (pH 5·0) per well was used as the enzyme substrate. Plates were incubated for 10 min in the dark, and the reaction was stopped with 100 μl of 0·01 M H_2SO_4 per well. The solutions were transferred to fresh ELISA plates and the optical densities were measured at 490 nm with an ELISA plate reader (Microplate Reader 450/550; Bio-Rad). The mAbs raised against *A. fumigatus* melanin were also tested for binding to other fungal melanins (from *C. neoformans*, *Penicillium marneffei*, *Sporothrix schenckii* and *A. niger*), synthetic melanin (Sigma) and melanin from *Sepia officinalis* (Sigma). For the assay, ELISA plates were coated with 50 μg of other fungal melanins, 50 μg of synthetic melanin and 100 μg of melanin from *Sepia officinalis*. Each ELISA was performed in triplicate and included a negative control which consisted either of uncoated wells

or coated wells in which the primary antibody was omitted. Anti-melanin *C. neoformans* mAb 6D2 (from Dr Josh Nosanchuk, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA) was used as a positive control.

Immunofluorescence (IF) analyses of melanin in *A. fumigatus* conidia. IF was performed on slide cultures of *A. fumigatus* and on melanin particles. Slide cultures and melanin particles were washed three times with PBS, then incubated with Superblock Blocking Buffer in PBS (Pierce) for 2 h at 37 °C or overnight at 4 °C to block non-specific binding. Samples were then washed three times with PBS and incubated with 10 mg ml^{-1} of the anti-melanin mAbs made up in Superblock blocking buffer in PBS for 1·5 h at 37 °C. After washing three times with PBS, slides and particles were incubated with a 1 : 100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories) for 1·5 h at 37 °C, washed three times with PBS and then mounted by using 50 % glycerol/50 % PBS. Cover slips were applied and samples were examined using a Zeiss immunofluorescence microscope. As a negative control, slides and particles were incubated with PBS in place of the primary antibody, followed by incubation with conjugated goat anti-mouse IgM as above.

Detection of laccase activity via non-denaturing gel electrophoresis. The laccase activity of cytoplasmic antigens from *A. fumigatus* was detected as described elsewhere (Wang *et al.*, 1995). The cytoplasmic antigens from *A. fumigatus* conidia and mycelia were extracted by using a homogenizer (Biospec) with 5 mm glass beads. The homogenate was centrifuged (10 000 *g* for 30 min), the pellet was discarded and the supernatant liquid was collected and concentrated by Amicron concentrators (molecular mass cut-off 5000) or by centrifugal filtration. Protein estimation was performed by the Coomassie brilliant blue method (Read & Northcote, 1981).Commercially available laccase [from *Rhus vernificera*, activity 50 U (mg solid)$^{-1}$] was obtained from Sigma. *R. vernificera* laccase (40 μg) and 250 μg of cytoplasmic conidial antigens of *A. fumigatus* B5233, NCPF 2010 and NCPF 2078 were loaded onto SDS-PAGE gels and, after electrophoresis, the gels were immersed in 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in 0·1 M citric acid/0·2 M Na_2HPO_4 (pH 6·0) buffer for 6–8 h. As a control, each of the above samples was boiled in water for 5 min to destroy any laccase activity, prior to loading onto the gel.

Isolation of melanin particles from human tissues and identification via IF reactivity with mAbs. Wax-embedded sections of human tissue from three patients with culturally confirmed nasal aspergilloma caused by *A. fumigatus* mounted on glass slides were subject to the melanin extraction protocol described above. Slides were deparaffinized in xylene and dehydrated by serial incubations in solutions of decreasing ethanol concentration. After the final treatment by boiling in acid, the slides were washed extensively with distilled water. Melanin particles were detected by IF analyses using anti-melanin mAbs and fluorescein isothiocyanate-conjugated goat anti-mouse IgM as described above.

RESULTS AND DISCUSSION

Melanin extraction from *A. fumigatus* conidia

Treatment of conidia with a combination of proteolytic and glycolytic enzymes, denaturant and hot, concentrated acid resulted in the isolation of black particles which were similar in size and shape to the original propagules as demonstrated by SEM (Fig. 1a, b). Clearly melanin is therefore deposited as a continuous 'shell' around the conidia. This was confirmed by TEM studies on the black particles (Fig. 1c) which identified electron-dense walls without internal structures

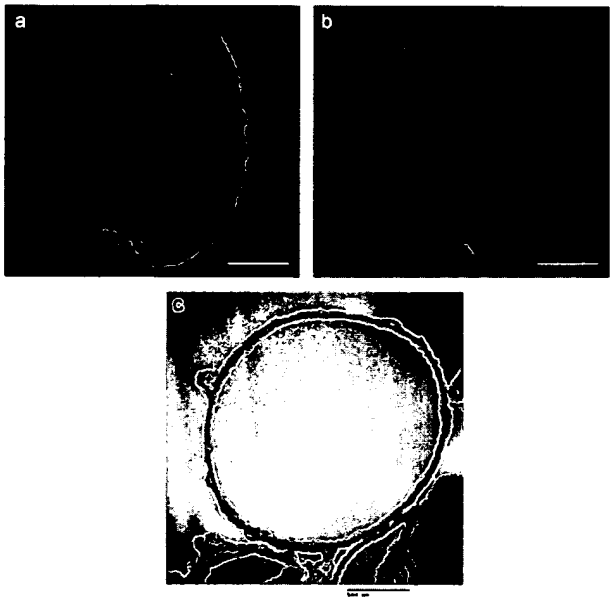

Fig. 1. SEM of *A. fumigatus* B5233 conidia before (a) and after (b) treatment with enzymes, denaturant and hot acid, and TEM of extracted particle (c). Bars, 0·5 μm.

(effectively hollow cells). The latter resembled those observed when melanin particles were extracted from *Sporothrix schenckii* yeast cells (Morris-Jones *et al.*, 2003). No hyphal structures were observed after *A. fumigatus* mycelia were subject to the melanin extraction protocol. *A. fumigatus* has previously been shown to produce pigment via the DHN-melanin pathway (Brakhage *et al.*, 1999; Langfelder *et al.*, 1998; Tsai *et al.*, 1998, 1999); much of this work has focused on the genes involved in biosynthesis rather than on the temporal and spatial characterization of pigment expression. The study described herein confirms via previously described chemical extraction methods (Gómez *et al.*, 2001; Morris-Jones *et al.*, 2003) that melanin is produced by *A. fumigatus* conidia.

ESR spectroscopy

ESR spectroscopy of the black particles collected from *A. fumigatus* conidia produced a signal indicative of a stable free-radical population consistent with the pigment being identified as a melanin (Fig. 2) (Enochs *et al.*, 1993). The spectrum was similar to the signal generated by the melanins extracted previously from *C. neoformans* (Rosas *et al.*, 2000a, b), *Paracoccidioides brasiliensis* (Gómez *et al.*, 2001), *H. capsulatum* (Nosanchuk *et al.*, 2002) and *Sporothrix schenckii* (Morris-Jones *et al.*, 2003), and represents the first biophysical confirmation of the production of melanin by *A. fumigatus*.

Generation of mAbs to *A. fumigatus* melanin

The successful extraction of melanin particles from

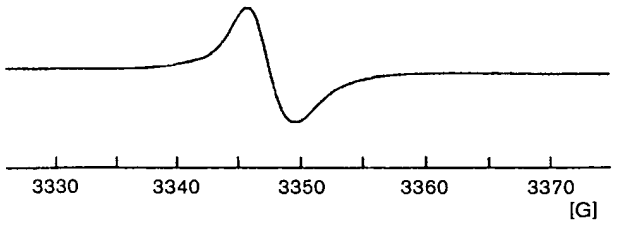

Fig. 2. ESR spectroscopy of melanin particles extracted from *A. fumigatus* B5233.

A. fumigatus conidia enabled the subsequent production, for the first time, of murine mAbs which were reactive with *A. fumigatus*-derived melanin. Mice were inoculated with melanin extracted from *A. fumigatus* B5233 conidia, and a high anti-melanin polyclonal response was induced (data not shown). After subcloning of hybridomas, a total of five anti-*A. fumigatus* melanin mAbs (8D6, 5C2, 8F5, 8G6 and 4C8) were produced. All these mAbs were of the IgM subclass and all demonstrated reactivity to fungal melanins extracted from the conidia of *A. niger*, *Sporothrix schenckii* and *Penicillium marneffei*, as well as to melanin extracted from yeast cells of *Sporothrix schenckii*. In addition, these mAbs bound L-DOPA melanin from *C. neoformans*, synthetic melanin and melanin from *Sepia officinalis* by ELISA (Fig. 3). Given that previous work has shown that the *A. fumigatus* melanin is almost certainly a DHN melanin, this would suggest that DHN melanin must share one or more epitopes with DOPA-melanin. This is an interesting observation given that so little is known about the antigenicity of melanin. These data were confirmed by the binding of the positive control antibody (anti-*C. neoformans* melanin mAb 6D2). No binding was observed in the negative control.

IF analyses

The anti-melanin mAb 8D6 bound to the surface of the melanin-like particles extracted from *A. fumigatus* conidia (Fig. 4) (as did the remaining four other mAbs and the positive control antibody 6D2; data not shown), which confirmed the distribution of this pigment within the conidial wall. The negative control (consisting of no primary antibody) was unreactive (data not shown). Slide cultures from *A. fumigatus* B5233 were examined at different time points using the anti-melanin mAb 8D6 by IF assay and demonstrated that biosynthesis of the pigment is restricted to the conidial and conidiophore stage (Fig. 5).The anti-melanin mAbs were strongly reactive to the conidial wall of freshly isolated conidia. However, as the conidia began to swell this reactivity lessened markedly and the uniform labelling was replaced by a patchy reactivity in the wall (Fig. 5c, d). Wall reactivity continued to decline during germination and disappeared completely firstly at the point from which the germ tube extended. This observation is analogous to that seen during budding in *C. neoformans* (Nosanchuk & Casadevall, 2003). In the latter, melanin degradation occurs in the cell wall in the immediate area from which the daughter cell will bud, leaving a bud scar. The controlled degradation of melanin within the *A. fumigatus* conidial wall must therefore be a crucial process which allows germination to occur, and the enzymic processes responsible are of great potential interest. The melanin synthesis genes then remain quiescent until conidiophore formation occurs – melanin extraction studies confirmed the complete absence of melanin in hyphae.

Detection of laccase-like activity in *A. fumigatus*

Conidial cytoplasmic extractions of *A. fumigatus* B5233, NCPF 2010 and NCPF 2078 were subject to PAGE in a non-denaturing gel and then incubated with 1 mM L-DOPA for

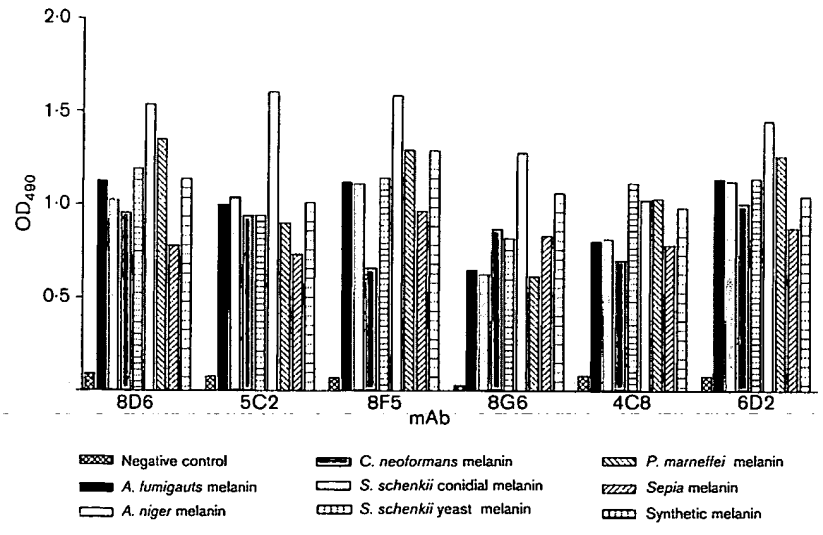

Fig. 3. ELISA reactivities of the five anti-*A. fumigatus* melanin mAbs and of mAb 6D2 (produced against melanin from *C. neoformans*), diluted 1 : 100, with the following eight types of melanins (50 µg per well): *A. fumigatus*, *A. niger*, *C. neoformans*, *Sporothrix schenckii* conidia, *Sporothrix schenckii* yeast, *P. marneffei* conidia, *Sepia* and synthetic melanin.

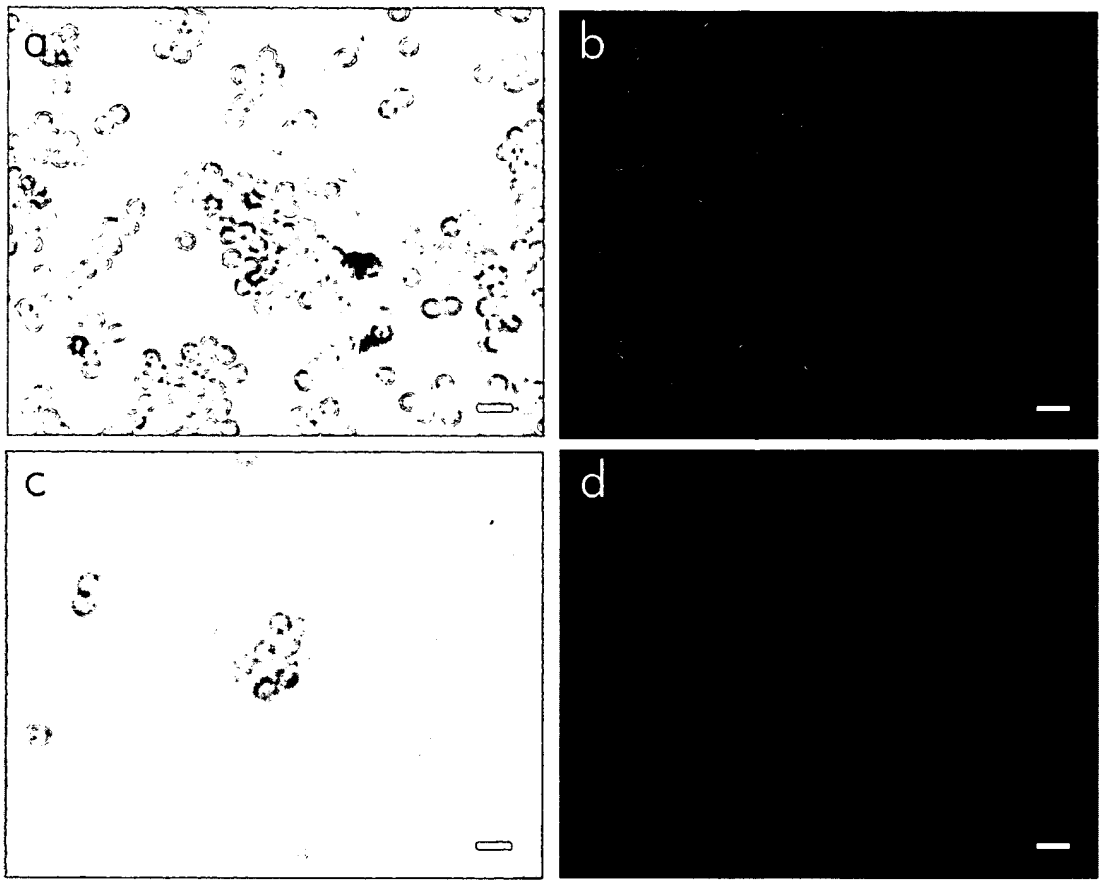

Fig. 4. Corresponding bright-field and IF microscopy images showing the labelling of *A. fumigatus* B5233 conidia (a, b) and the melanin particles derived from *A. fumigatus* B5233 conidia (c, d) by anti-melanin mAb 8D6. Bars, 5 μm.

6 h. Positive laccase activity was revealed by dark bands conforming to the polymerization of L-DOPA-melanin. Laccase-like activities which were comparable to that produced by the positive control were found in conidial cytoplasmic extracts from all isolates of *A. fumigatus* (Fig. 6). Boiling the samples in water for 5 min abrogated the laccase activities (Fig. 6). The cytoplasmic extractions from *A. fumigatus* hyphae showed no laccase-like activity (data not shown). These observations are consistent with previous work that found a laccase-encoding gene in *A. fumigatus* (*abr2*), although it is not yet clear at which point of the melanin biosynthetic pathway this enzyme(s) is involved (Tsai *et al.*, 1999). Specifically, functions should be assigned to two genes in the *A. fumigatus* gene cluster, *abr1* (a putative multicopper oxidase) and *abr2* (a putative laccase). The involvement of the two genes in the formation of the bluish-green conidial colour was shown by disruption of each gene which led to altered conidial colour phenotypes (brown conidia) (Tsai *et al.*, 1999). However, hyphal cytoplasmic antigens were negative for laccase-like activity which confirms the absence of melanin shown using the anti-melanin mAbs.

Extraction of melanin particles from human tissue infected with *A. fumigatus*

Treatment of human nasal aspergilloma with denaturant, enzymes and hot, concentrated acid yielded a small quantity of dark material which when observed microscopically contained particles that were the same size and shape as conidia of *Aspergillus* (Fig. 7). These particles were reactive by IF with anti-melanin mAb 8D6, whereas particles incubated with the fluorescein conjugate alone did not show any reactivity (data not shown). No hyphal material was seen. This represents the first indication that melanin may be formed during the course of *Aspergillus* infection. However, a previous study has shown that expression of the *pksP* gene, which is involved in the biosynthesis of bluish-green conidial pigment, can be detected in hyphae of germinating conidia isolated from the lungs of immunocompromised mice (Langfelder *et al.*, 2001). Typically, conidiation does not occur during disseminated aspergillosis infections, but it may happen during lung and nasal infection (i.e. during aspergilloma formation). Our data would suggest that conidiation *in vivo* is accompanied by melanization. Given that melanized conidia

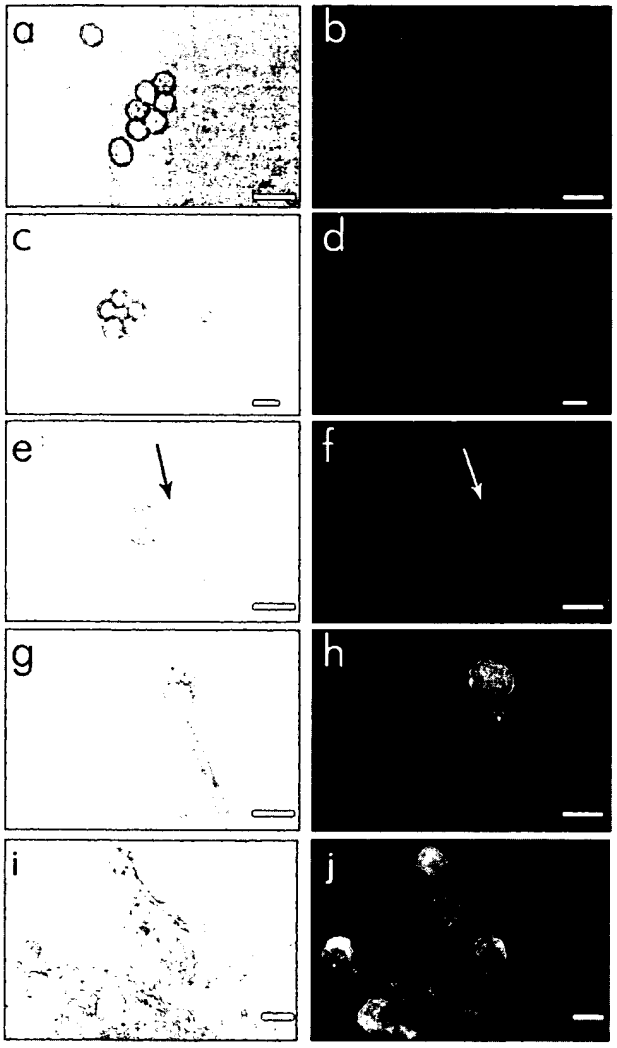

Fig. 5. Corresponding bright-field and IF microscopy images demonstrating the labelling of the germination of *A. fumigatus* B5233 at different time points: at day 0 (a, b); 1 day old (c, d); 2 days old (e, f); 5 days old (g, h); and 7 days old (i, j). The arrow indicates germ tube of conidia. Bars, 5 μm.

have been shown to be resistant to attack by reactive oxygen species (Jahn *et al.*, 1997, 2000), it may be that these propagules play a role in maintaining infection during aspergilloma formation and development.

In summary, our data revealed that *A. fumigatus* conidia synthesized melanin-like pigments both *in vitro* and *in vivo*. We confirmed using biophysical methods that the dark pigment extracted from *A. fumigatus* conidia was melanin. Novel anti-melanin mAbs were produced and used to examine the expression of this pigment during development. Further studies will be directed towards a more detailed analysis of the processes underlying the degradation of melanin during germination.

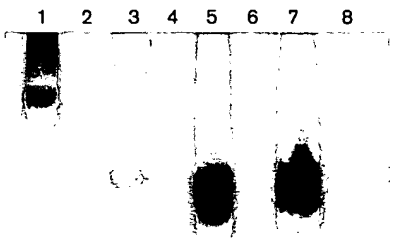

Fig. 6. Non-reducing SDS-polyacrylamide gel of cytoplasmic antigen extract of *A. fumigatus* developed with L-DOPA. Lanes: 1, commercial laccase (40 U equivalent); 2, as for 1 but boiled for 5 min; 3, 250 μg of conidial antigen of *A. fumigatus* B5233; 4, as for 3 but boiled for 5 min; 5, 250 μg of conidial antigen of *A. fumigatus* NCPF 2010; 6, as for 5 but boiled for 5 min; 7, 250 μg of conidial antigen of *A. fumigatus* NCPF 2078; 8, as for 7 but boiled for 5 min.

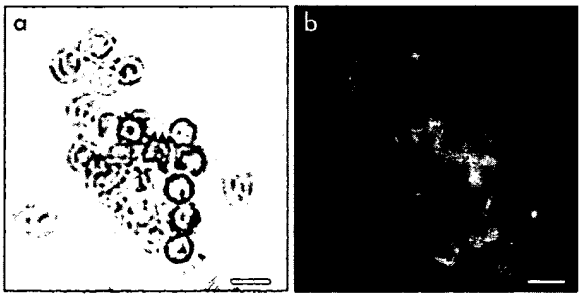

Fig. 7. Corresponding bright-field (a) and IF microscopy (b) images of melanin particles extracted from nasal aspergilloma in human tissue labelled by anti-melanin mAb 8D6. Bars, 2 μm.

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